Demonstration of an unidentified 48 kD polypeptide in circulating immune complexes in rheumatoid arthritis

R D MELSOM, P R SMITH, AND R N MAINI

From the Kennedy Institute of Rheumatology, 6 Bute Gardens, Hammersmith, London

SUMMARY   Circulating immune complexes (CIC) were isolated from 25 patients with rheumatoid arthritis (RA) by anti-C1q affinity chromatography. The components were detected by silver stained sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and identified by the Western blot. The CIC were composed of 20 different polypeptides, including albumin, immunoglobulin, complement, and acute phase reactants. Two components (molecular weight 48 kD and 45 kD respectively) remained unidentified. The 48 kD polypeptide was found in CIC from six out of 14 patients (43%) with extra-articular RA, but from none of eight patients with vasculitic complications of other connective tissue diseases. All immunoreactants were more frequently found in the patients with extra-articular features of RA. Although these results emphasise that most CIC in RA are composed of endogenous proteins, the 48 kD polypeptide is a candidate for an extrinsic antigen in RA.

Key words: silver stain, Western blot, antigen.

Immune complexes (IC) have been isolated from synovial fluid of patients with rheumatoid arthritis (RA) and extensively analysed.[1][2] No specific antigen other than IgG complexed to rheumatoid factor has been found. Circulating immune complexes (CIC) also occur in RA,[3] but they have been subject to only limited analysis after prior isolation.[4][5] In our laboratory C1q binding CIC have been measured and found in the greatest quantity in patients with extra-articular features of RA.[6-8][8a] Others have isolated C1q binding CIC by anti-C1q affinity chromatography.[9] We used this technique combined with specific and sensitive detection techniques to identify the components of CIC found in RA, particularly those with extra-articular features.

Patients and methods

PATIENTS
Serum was collected and separated at 37°C to avoid loss of CIC by cryoprecipitation from five normal

subjects, 25 patients with classical or definite RA,[10] and eight patients with other connective tissue diseases. All the patients with RA had active disease and 14 had extra-articular disease, including the following features: rheumatoid nodules (10 patients), vasculitis (10), and Sjögren's syndrome (SS; seven). The patients without RA included two with systemic lupus erythematosus (SLE), each with four of the new SLE criteria,[11] five with SS and extraglandular features, and one with an undifferentiated connective tissue disease. Patients with SS had restricted tear flow in either eye of less than 5 mm wetting of a sterile strip (Cooper Health Products Ltd, Aylesbury, Bucks, UK).

PREPARATION OF ANTI-C1q —
IMMUNOSORBENT
Anti-C1q antiserum was raised in rabbits using C1q isolated by a previously described method.[12] The IgG anti-C1q fraction was isolated from rabbit serum by precipitation with 50% saturated ammonium sulphate followed by protein A Sepharose (Pharmacia Fine Chemicals, Milton Keynes, UK) chromatography. Antiglobulins were removed by passage three times through a 20 ml normal (non-

immune) rabbit IgG (Nordic, 2 St Peter's Road, Maidenhead, UK) Sepharose affinity chromatography column. The anti-C1q antisera formed one line of precipitation on immunodiffusion in 0·5% agarose (Miles Scientific) against both C1q and normal human serum. The two precipitins showed complete identity. Anti-C1q was coupled to CNBr activated Sepharose 4B (Pharmacia) at 10 mg of protein ligand/ml of Sepharose.

ISOLATION OF CIC

CIC were isolated by a combination of 2% polyethylene glycol (PEG) precipitation[13] and anti-C1q affinity purification. Serum (2·5 ml) was brought to a final concentration of 2% PEG, mol. wt 6000, in 0·1 M veronal buffered saline, pH 7·6, containing 0·06 M ethylenediaminetetra-acetate. Precipitates which formed overnight at 4°C were centrifuged at 2000 g for 20 minutes at 4°C, washed once with 2·5 ml of 2% PEG, and recentrifuged. The final precipitate was redissolved in 2·5 ml of phosphate buffered saline (PBS) at 37°C for one hour with intermittent agitation. An aliquot (2 ml) of the resolubilised PEG precipitate was applied to a 5 ml affinity chromatography column equilibrated in PBS at a flow rate of 10 ml/hour. After washing, bound complexes were eluted by two complete cycles (4 ml each) of 0·1 M glycine, pH 2·5, and 0·1 M sodium bicarbonate, pH 8·3, both containing 0·5 M NaCl. The eluate was dialysed extensively against PBS and concentrated by vacuum dialysis to approximately 1 ml. IgM, IgG, and IgA were measured in the eluate by radial immunodiffusion.[8a]

SDS-PAGE AND IMMUNOBLOTTING

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described previously.[14] Affinity purified CIC (20 μl) or approximately 5–20 μg of other proteins were applied to each well. In some cases affinity purified CIC were concentrated by incubation in 20% trichloroacetic acid at room temperature for 10 minutes and centrifuged at 12 000 g for 10 minutes. The precipitates were washed in acetone and redissolved in reducing sample buffer before electrophoresis. Gels were stained with 0·05% Coomassie blue R250, destained, and then silver stained (Bio-rad Laboratories).

For Western blotting, components were transferred by electrophoresis onto nitrocellulose sheets and remaining active sites blocked by incubation in 5% bovine serum albumin (Sigma, Fancy Road, Poole, Dorset, UK) in PBS for one hour at 37°C. The nitrocellulose was incubated for one hour with antisera or patient's sera diluted 1:500 or 1:100 respectively in blocking solution, and bound antibody visualised using ^{125}I labelled protein A. The Western blot was developed with commercially available polyclonal antisera to IgM, IgG, IgA, IgE, albumin, C1q, C1s, C3c, C3d, C4, C9, fibrinogen, C reactive protein (CRP), α_2 macroglobulin, or whole normal human serum. CIC were probed with one rheumatoid serum with high titre antibody to rheumatoid arthritis nuclear antigen (RANA), and one without this antibody as assessed by immunoblotting[15] and immunodiffusion.[16] Each gel contained at least one normal sample.
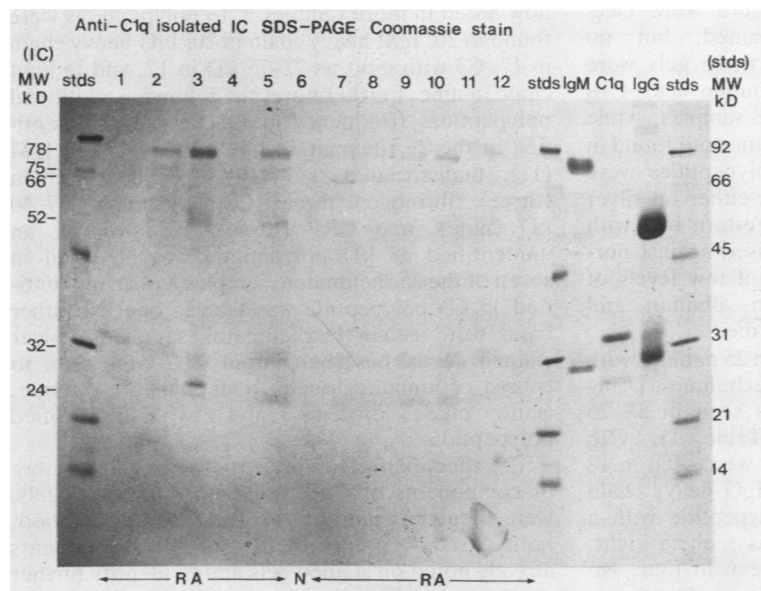

Fig. 1 *Molecular weight standards (stds), IgM, C1q, IgG, and CIC isolated from 11 patients with RA (lanes 1–5 and 7–12) and one normal (lane 6) were stained with Coomassie brilliant blue. The molecular weights of the standard proteins and of the major components of the CIC are shown on the right and left sides of the figure respectively.*

Table 1 *Proteins identified in CIC isolated from patients with RA*

Protein	Mol. wt (kD)	Coomassie stained SDS-PAGE*	Silver stained SDS-PAGE*	Western blot*
IgM (undissociated)	500		11/25	
IgG (undissociated)	150		10/25	
C3	112			3/12
C4	82			5/12
C1s	83			3/8
IgM heavy chain	78	16/25	20/25	13/13
C4	78			7/12
C3	75	}8/25†	}17/25†	7/12
C3	72			7/12
Albumin	66	25/25	25/25	
IgA heavy chain	62		3/25	3/9
Fibrinogen	58		3/25	3/9
IgG heavy chain	52	11/25	17/25	7/9
Unidentified	48		7/25	
Unidentified	45		1/25	
C3	40		}9/25†	5/12
C3	37			3/12
C4	33			3/12
C1q	32 (doublet)	18/25	20/25	13/13
Immunoglobulin light chain	25	4/25	9/25	
C1q	24 (doublet)	18/25	20/25	4/13
C reactive protein	26		3/25	3/9

*Figures represent the number of samples containing each component in relation to the number of samples tested.
†By this technique it was not possible to distinguish between the polypeptides of different mol. wt.

Results

Dilutions of IgG and C1q were added to individual lanes of SDS-PAGE gels, electrophoresed, and stained with Coomassie brilliant blue. This method was able to detect 20 ng of IgG and 175 ng of C1q. CIC isolated from five normal sera were electrophoresed and Coomassie stained, but no polypeptides were visible. When these gels were destained and silver stained, albumin and C1q polypeptides were seen in all five samples, while IgM heavy chain and IgG heavy chain were found in three samples each. No other polypeptides were detected in these normal samples, either on silver stained gels or when reacted in Western blot with specific antisera, including that raised against normal human serum. The presence of low levels of CIC consisting of immunoglobulin, albumin, and C1q in normal sera was thus confirmed.

Components of CIC isolated from 25 patients with RA were then examined by these techniques. Using the Coomassie stain albumin was seen in all 25 rheumatoid samples (Fig. 1, Table 1). C1q polypeptides and IgM heavy chain were seen in 18 and 16 preparations respectively, IgG heavy chain was present in 11, and a C3 polypeptide with a molecular weight of 72–75 kD was seen in eight. Immunoglobulin light chain was seen in four. No other polypeptides were seen by this method.

The gels were then silver stained, a method already shown to demonstrate components in CIC from normals when none could be seen on Coomassie stain. Albumin was again present in all 25 rheumatoid samples (Fig. 2). The polypeptides previously seen on the Coomassie stained gels were now noted in more samples. C1q polypeptides were found in 20, IgM heavy chain in 20, IgG heavy chain in 17, C3 with mol. wt 72–75 kD in 17, and Ig light chain in nine. Furthermore, the following additional polypeptides (frequency in brackets) were identified in the 25 rheumatoid CIC: undissociated IgM (11), undissociated IgG (10), IgA heavy chain (three), fibrinogen (three), C3 with mol. wt 37–40 kD (nine), and CRP (three). Additionally, an unidentified 48 kD polypeptide was observed in seven of the 25 rheumatoid samples and an unidentified 45 kD polypeptide was seen in one. No other bands were seen in the rheumatoid samples on silver stained gels. Thus rheumatoid CIC were seen to consist of immunoglobulin, complement, albumin, acute phase proteins, and two unidentified polypeptides.

The rheumatoid samples with the largest number of components of CIC, as noted on stained gels, were then examined by the Western blot method, both to confirm the identity of the components already noted on stained gels and to identify further components (Fig. 3). This technique allowed the
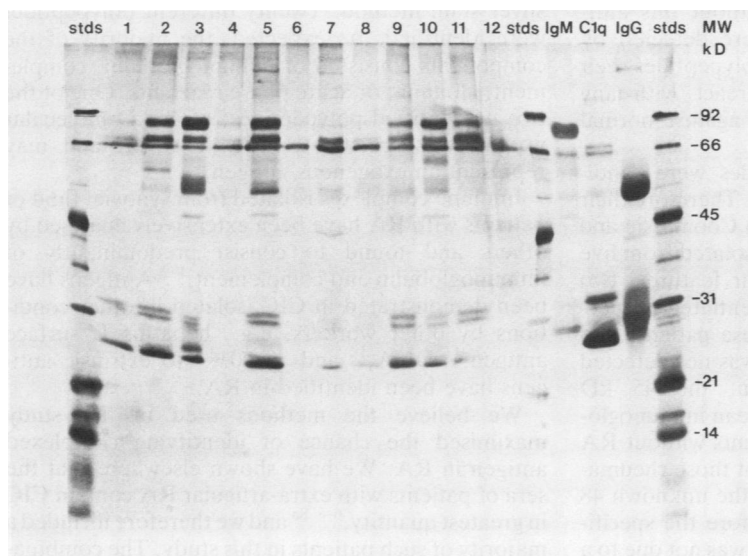
Fig. 2 *The gel seen in Fig. 1 was destained and then silver stained. Molecular weights of the standard proteins are shown on the right.*

detection of 1 ng of IgG and 11 ng of C1q. By this method the following polypeptides were seen at a greater frequency (shown in brackets) than on the stained gels; IgM heavy chain (13/13), IgA heavy chain (3/9), fibrinogen (3/9), IgG heavy chain (7/9), CRP (3/9), and the 32 kD C1q doublet (13/13). The 24 kD C1q doublet was seen in 4/13 samples. The following additional components (frequency in brackets) were also detected by immunoblotting: C3 of mol. wt 112 kD (3/12), C4 of mol. wt 82 kD (5/12), C1s (3/8), C4 of mol. wt 78 kD (7/12), and C4

of mol. wt 33 kD (3/12). By the Western blot the C3 polypeptide, noted as a band of mol. wt 72–75 kD on the stained gels, was seen to consist of separate polypeptides of 75 kD (in 7/12) and 72 kD (in 7/12) samples. Similarly the 37–40 kD C3 band was noted to represent two polypeptides of 37 kD (in 3/12) and 40 kD (in 5/12) respectively. Twelve rheumatoid samples were blotted with antisera against IgE, C9, and α_2 macroglobulin without detection of the corresponding antigen. They were also reacted with serum from a patient with RA and high titre
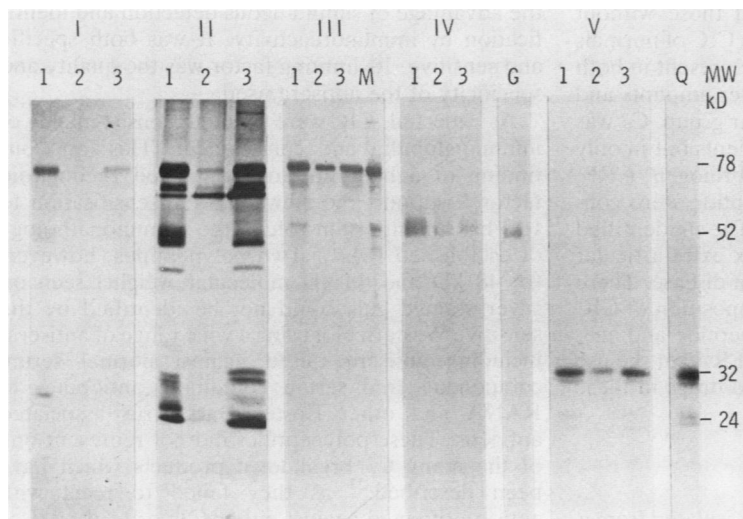

Fig. 3 *Twenty microlitres of the CIC from three patients with RA (lanes 1–3) were electrophoresed on SDS-PAGE gels and Coomassie stained (I), silver stained (II), or developed in the Western blot with anti-IgM (III), anti-IgG (IV), or anti-C1q (V) antisera. On some gels, IgM (M) IgG, (G), and C1q (Q) were also electrophoresed. Molecular weights of the major components are shown on the right.*

anti-RANA antibody, and one without this antibody. No RANA polypeptides were detected by these sera. The 48 kD and 45 kD polypeptides seen on silver stained gels failed to react with any antisera, including antisera raised against normal human serum proteins.

The two unidentified polypeptides were candidates for an extrinsic antigen in RA. Therefore their disease specificity was compared on Coomassie and silver stained gels with that of CIC isolated from five patients with SS and extraglandular features, two with SLE, and one with an undifferentiated connective tissue disease. All eight of these patients had vasculitis. The 48 kD polypeptide was not detected in the patients without RA, but the 45 kD polypeptide was seen in two. The mean immunoglobulin level in CIC in the eight patients without RA (44 mg/ml) was nearly double that of those rheumatoid patients whose CIC contained the unknown 48 kD polypeptide (27 mg/ml). Therefore the specificity of the 48 kD polypeptide to RA was not due to a concentration phenomenon. The CIC of the 14 rheumatoid patients with the most complexed material and of all eight patients without RA were then precipitated with trichloroacetic acid and more protein added to the gels to see whether smaller quantities of the 48 kD polypeptide could be found in either group. This did not alter either the frequency of detection or the disease specificity of the 48 kD polypeptide.

We then considered the appearance of CIC oh silver stained gels, and particularly the presence of the unidentified 48 kD polypeptide, in relation to the clinical features of the patients with RA. A difference was noted between those with extra-articular features (14 patients) and those without (11). The components present in the CIC of normals (IgM, IgG, C1q, and albumin) were present in both rheumatoid groups, though in greater amounts and more frequently in the extra-articular group. C3 was detected in 11/14 extra-articular patients but in only 6/11 with articular disease. IgA, fibrinogen, CRP, and the unidentified 45 kD polypeptide were confined to the extra-articular group. The unidentified 48 kD polypeptide was found in six extra-articular patients but in only one with articular disease. There was no correlation between the composition of CIC or the presence of the 48 kD polypeptide and age, sex, disease duration, age at onset of RA, or the use of disease modifying antirheumatoid drugs in these patients.

Discussion

This is the first study to examine CIC isolated from a large number of patients with RA by the sensitive silver stain method. Twenty different polypeptides were identified. As expected, the majority of the components consisted of immunoglobulin, complement, albumin, or acute phase reactants. One of the two unidentified polypeptides, of 48 kD molecular weight, was specific to RA, however, and may represent an exogenous antigen.

Immune complexes isolated from synovial fluid of patients with RA have been extensively analysed by others and found to consist predominantly of immunoglobulin and complement.[1] [2] Antigens have been demonstrated in CIC isolated in other conditions by other workers, e.g., hepatitis B surface antigen,[17] DNA,[18] and gp 70.[19] No extrinsic antigens have been identified in RA.

We believe the methods used in this study maximised the chance of identifying complexed antigen in RA. We have shown elsewhere that the sera of patients with extra-articular RA contain CIC in greatest quantity,[6-8] [8a] and we therefore included a majority of such patients in this study. The combination of a preliminary 2% PEG precipitation and anti-C1q affinity chromatography ensured isolation of CIC with minimal contamination from other serum constituents, e.g., uncomplexed C1q and immunoglobulin.[20] The Coomassie stain was useful only to compare components present in large amounts. The silver stain had the advantages of greater sensitivity,[21] and has been used by others to identify up to 25 components in CIC isolated from patients with SLE.[22] We found it the most useful method of screening CIC for their constituents. Components provisionally identified by molecular weight alone on silver stained gels were then examined by immunoblotting. The Western blot had the advantage of simultaneous detection and identification by immunoreactivity. It was both specific and sensitive. Its limiting factor was the quality and specificity of the antisera used.

As expected, CIC were found to consist mainly of immunoglobulin and complement. This, and our finding of a high titre of complexed rheumatoid factor,[8a] support the view that autosensitisation to IgG has an important role in the immunopathology of established RA.[1] [2] Two polypeptides, however, (of 48 kD and 45 kD molecular weight) seen on silver stained gels could not be identified by the sensitive Western blot using a wide range of antisera, including antisera raised against normal serum components and serum containing antibodies to RANA and other Epstein-Barr virus associated antigens. These polypeptides did not represent one of the many C3 breakdown products which have been described,[23] as they failed to react with antisera directed against either C3c or C3d.

The 45 kD polypeptide was present in a small

minority of samples and lacked disease specificity, unlike the 48 kD polypeptide, which was confined to RA. The latter was found in nearly 50% of the extra-articular group, where complexed antigen would be predicted to occur. This 48 kD polypeptide may represent an exogenous antigen, and its identification might therefore shed light on the aetiology of rheumatoid arthritis.

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